IN VITRO PRODUCTION OF ERGOT ALKALOIDS BY CULTURES OF CLAVICEPS PURPUREA (FR.) TUL.[1]

W. A. TABER AND L. C. VINING

Abstract

Stationary cultures of *Claviceps purpurea* (Fr.) Tul. grown 50 days on a liquid medium containing mannitol, ammonium succinate, and salts produced small amounts of ergot alkaloids.

Ergometrine, ergometrinine, ergotamine, ergotaminine, ergocornine, ergocorninine, ergocryptine, agroclavine, and penniclavine were identified by paper chromatography of culture extracts. The selection of strains, the composition of the medium, and the length of the incubation period appeared to be important factors influencing the production of alkaloids.

Introduction

Since 1930 the production of ergot alkaloids by pure cultures of *Claviceps purpurea* has frequently been reported (8, 9, 10, 13, 14, 15, 18, 19, 22) but in most cases the methods employed for the identification of the alkaloids were not exacting and depended solely upon characteristic biological properties and color reactions. Production, if actually achieved, was at a very low level. Many recent workers (11, 12, 17, 20, 24) have failed to reproduce these results and, in fact, the majority of reports since 1950 have indicated that alkaloids are not produced by the fungus *in vitro*. Abe and his coworkers (1 to 8), and subsequently Stoll and his group (22) using Abe's methods, have, however, demonstrated convincingly that alkaloid production is possible. Where high yields were obtained, the products were found to be not the substituted amides of lysergic acid hitherto isolated from rye sclerotia but new derivatives of the ergoline ring system. Thus Abe's group have found that certain cultures isolated from *Agropyron semicostatum* Nees produced good yields of agroclavine (1), while cultures from *Elymus mollis* Trin. produced substantial quantities of elymoclavine (7). A number of other alkaloids of novel constitution have also been isolated in minor amounts. Stoll *et al.* (22) described the isolation of penniclavine, elymoclavine, and agroclavine from a strain of *Claviceps* found on *Pennisetum typhoideum* Rich. Recently, the Japanese workers (8) have reported paper chromatographic evidence that small amounts of ergometrine, ergotamine, ergosine, ergocristine, ergokryptine, and ergocornine and of alkaloid X, agroclavine, and elymoclavine are produced *in vitro* by a Spanish strain of *Claviceps purpurea*. Strains isolated from 10 different grasses were examined, and all cultures were found to produce agroclavine, elymoclavine, alkaloid X, ergokryptine, and ergosine; two produced ergometrine; three, ergotamine; three, ergocristine; and one, ergocornine.

In view of the importance of ergot alkaloids and the obvious advantages of commercial production in artificial media, an investigation into the possibili-

---

[1] Manuscript received November 5, 1956.

Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.

Issued as N.R.C. No. 4183 and as Paper No. 237 on the Uses of Plant Products.

ties of *in vitro* production was undertaken. This phase of the study was designed to resolve the conflict between recent reports in the literature concerning the formation or nonformation, in artificial media, of ergot alkaloids of the type derived from lysergic acid.

Materials and Methods

Growth Conditions

The history, nutrition, and method of preparing the inocula of the three strains used in these studies have been described in a previous publication (23). The influence of media composition on production was examined by the use of five different media (Table I).

These media were dispensed either in 40 ml. aliquots in 125 ml. Erlenmeyer flasks, or 50 ml. in 250 ml. Erlenmeyer flasks. Shaken cultures were grown as already described (23); stationary cultures were incubated at 27° C. either in the dark or diffuse natural light.

Estimation of Yield of Alkaloid

Cultures were selected at intervals, separately homogenized in a Waring blendor, and freeze-dried. They were then shaken for four hours with peroxide-free ether (50 ml.) containing concentrated ammonium hydroxide (0.3 ml.), filtered, washed with fresh ether (20 ml.), and the filtrate concen-

TABLE I

COMPOSITION OF MEDIA

	Concentration per liter				
	Medium I*	Medium II	Medium III†	Medium IV	Medium V
Glucose		10 g.	15 g.		50 g.
Mannitol	50 g.			15 g.	
Yeast extract		10 "			
Ammonium succinate	8 "		980 mg.	980 mg.	8 "
K_2HPO_4			100 "	100 "	100 mg.
KH_2PO_4	1.0 "		700 "	700 "	700 "
$MgSO_4.7H_2O$	0.3 "		201 "	201 "	201 "
$CaCl_2.2H_2O$			5.95 "	5.95 "	5.95 "
$FeSO_4.7H_2O$			5.00 "	5.00 "	5.00 "
$ZnSO_4.7H_2O$			4.40 "	4.40 "	4.40 "
$MnSO_4.H_2O$			2.75 "	2.75 "	2.75 "
NaCl			2.55 "	2.55 "	2.55 "
$CuCl_2.2H_2O$			0.269 "	0.269 "	0.269 "
$(NH_4)_6Mo_7O_{24}.4H_2O$			1.82 "	1.82 "	1.82 "
i-Inositol			1.0 "	1.0 "	1.0 "
Thiamine HCl			100 μg.	100 μg.	100 μg.
Pyridoxine HCl			100 "	100 "	100 "
Calcium pantothenate			100 "	100 "	100 "
Nicotinamide			50 "	50 "	50 "
p-Aminobenzoic acid			50 "	50 "	50 "
Biotin			5 "	5 "	5 "

*Reference 1. Final medium, in tap water, was adjusted to pH 5.2–5.4 with succinic acid. The other media were made up in distilled water.

†Reference 23.

trated at 40° C. under a stream of nitrogen to approximately 10 ml. This was shaken with 3 ml. of 0.1 *N* sulphuric acid and the aqueous layer separated. To it was added 6 ml. of Van Urk's reagent (*p*-dimethylaminobenzaldehyde (0.2 g.) and $FeCl_3.6H_2O$ (0.003 g.) in 100 ml. 65% (v/v) sulphuric acid). After one hour, the color of the sample was measured in a Coleman Junior Spectrophotometer at 550 mμ and the amount of alkaloid calculated as ergometrine was determined from a previously prepared standard curve.

Recovery and Identification of Products

Separation of the alkaloids was carried out by a modification of the method used by Stoll and Rüegger (21). The whole cultures were freeze-dried and shaken twice with 500 ml. petrol (b.p. 30°–60° C.) to remove the bulk of the fats, and the residue extracted once with 250 ml. of 2% tartaric acid in 70% aqueous acetone, followed by three further extractions of 100 ml. each. The extracts were concentrated at 40° C. *in vacuo* to remove the acetone, then extracted twice with 100 ml. of ether, and the extract discarded. The aqueous solution was neutralized with sodium carbonate, then extracted four times with 100 ml. of a mixture of chloroform and isopropanol (3 : 1). The extracts were concentrated *in vacuo* to a small volume (0.1 ml.), and a sample of the solution examined by circular paper chromatography. A disk of Whatman No. 1 filter paper (24 cm. diameter) was dipped in a mixture of equal volumes of formamide and ethanol (16) and well blotted. In order that the ethanol might evaporate, at least 15 min. was allowed to elapse after application of the alkaloid samples before the paper was placed in a large desiccator and irrigated with a solvent mixture consisting of four parts of benzene to one part of petrol (b.p. 100°–120° C.). The solvent front took approximately two hours to reach the outer edge of the paper. The air-dried chromatogram was examined under ultraviolet light of 2537 Å, and fluorescent zones noted. The paper was then sprayed with a 2% (w/v) solution of *p*-dimethylaminobenzaldehyde dissolved in concentrated hydrochloric acid. Dark blue zones appeared on the chromatogram within a few minutes. A second sample of the culture extract was then chromatographed in the same manner using chloroform saturated with formamide as the mobile phase. Using the solvent mixture benzene–petrol (4 : 1) on formamide impregnated paper, it was found that a mixture of the following alkaloids could be separated, in order of increasing R_f value: ergometrine, ergotamine, ergosine, ergotaminine, ergosinine, ergocornine, ergokryptine, ergocorninine, ergocristinine, and ergokryptinine. Ergocristine appeared between ergosinine and ergocornine, but since these two alkaloids themselves were very close together, inclusion of ergocristine in the mixture resulted in a single broad band in which the three individual components were indistinguishable. Ergosine and agroclavine were superimposed on ergotamine, while ergometrinine, elymoclavine, and penniclavine were superimposed on ergometrine in the solvent system. When the system chloroform–formamide was used, a mixture of penniclavine, ergometrine, ergometrinine, agroclavine, and ergotamine could be separated (in order of increasing R_f). Elymoclavine was superimposed on ergometrine, while all the remaining alkaloids were superimposed on ergotamine at the solvent front in this system. Identification of the alkaloids in the culture samples was carried out by comparison of the zones with a mixture of standards in an adjacent segment of the chromatogram. Only those zones which gave a blue fluorescence in ultraviolet light as well as a blue or green zone with Van Urk's reagent were recorded, except with agroclavine and elymoclavine, which do not fluoresce.

Results

When the three cultures were grown on medium I under stationary conditions and sampled after intervals of 25, 35, and 52 days, no alkaloid could be detected until the third sampling, when 0.51 to 1.5 mg. per liter of culture (calculated as ergometrine) were found in PRL 1555 and 1557. No alkaloid was found in PRL 1556. Duplicate experiments were carried out under conditions of dark and light and with added biotin (23), but no marked differences were noted. A positive color reaction, equivalent to 0.53 mg. of ergometrine per liter, was obtained at 35 days when shake cultures of PRL 1555 in medium I were used.

The influence of different media on production was tested by growing the three strains on the five media described and examining the cultures at 48 days. PRL 1555 alone was found to produce alkaloids in this experiment. PRL 1556 and 1557 gave no color reaction with Van Urk's reagent even when re-examined after 65 days. Medium I gave the best yield of alkaloid (0.55 mg. per liter culture) but some production also occurred on medium III (0.25 mg.) and II (the estimated value of 0.52 mg. obtained here was undoubtably too high owing to the presence of a brown pigment extracted along with the alkaloids). No appreciable production occurred on media IV and V. Paper chromatograms of extracts from five pooled flasks of PRL 1555 grown on medium I showed the presence of ergometrine and ergotamine.

TABLE II

ALKALOIDS FORMED IN STATIONARY CULTURES OF *Claviceps purpurea**

PRL 1555	PRL 1557
Penniclavine	Ergometrine (and possibly elymoclavine)
Ergometrine (and possibly elymoclavine)	Agroclavine
Ergometrinine	Ergotamine
Agroclavine	Ergocornine
Ergotamine	Ergokryptine
Ergotaminine	Ergocorninine
Ergocornine	
Ergokryptine	

*Fifty-day-old submerged culture on Abe's medium (1).

When PRL 1555 and 1557 were grown under stationary conditions for 50 days and the alkaloids from 20 pooled flasks of each were isolated and examined by paper chromatography, alkaloids were again detected (Table II).

Discussion

The most notable differences in the conditions for alkaloid production used by Abe and his group, compared with those used by other workers, are the long incubation periods (30–50 days), the use of a medium consisting of 5% mannitol, 0.8% ammonium succinate, 0.1% monopotassium phosphate, 0.03% magnesium sulphate, and tap water, and the adjustment of the pH of the medium (with succinic acid) to pH 5.2–5.4. Under these conditions the formation of alkaloids by two of the three strains of *Claviceps* used was shown to take place and Abe's results confirmed. Although production at this level is of no commercial significance, it does serve to demonstrate that certain strains of *Claviceps* possess the ability to synthesize the lysergic acid molecule *in vitro*, and hence do not need to rely either on preformed nutrients from the host plant or formation of sclerotia. It seems reasonable to expect that further experiments may reveal conditions which will allow a significant increase in yield.

The work carried out up to the present indicates that three conditions are important. The first is selection of a proper strain. Of the three used above, one (PRL 1556) gave no production and a second (PRL 1557) gave only irregular production. Abe *et al.* (8) have also shown that strains vary in the types of alkaloid produced and it is of interest that the alkaloids detected in the *in vitro* cultures were also found in the sclerotia from which they were isolated. The composition of the medium is also important. Abe's medium was superior to all others tested, although it does not appear to be essential for production. Stoll *et al.* (22) also found that their culture would produce alkaloids on other media, and that production on Abe's medium could be improved by the addition of 5% sucrose. Finally, a long period of incubation is necessary. Our results indicate that at least 35 days are required for production when shake cultures are used and longer periods for stationary cultures. Stoll *et al.* (22) found 20 days were required for detectable production with shaken cultures and 42 days with stationary cultures.

The numerous reports of failure by other investigators to achieve alkaloid production is understandable since few, if any, of the above conditions were observed in their experiments.

Acknowledgments

The authors wish to thank Mr. F. M. Clare, Miss M. J. A. Gates, and Miss C. A. Marshall for technical assistance, and Dr. G. A. Ledingham for advice and encouragement, as well as the gift of samples and cultures. The gifts of samples of alkaloids by Sandoz Ltd., Basle, Switzerland, and by Dr. C. A. Morell of the Food and Drug Division, Department of National Health and Welfare, Ottawa, are also gratefully acknowledged.

References

1. Abe, M. Researches on ergot fungus. Parts III–XVII. Ann. Repts. Takeda Research Lab. **10**, 73–234 (1951).
2. Abe, M., Yamano, T., Kozu, Y., and Kusumoto, M. Researches on ergot fungus. Part XVIII. Production of ergot alkaloids in submerged cultures. J. Agr. Chem. Soc. Japan, **24**, 416–422 (1951).
3. Abe, M., Yamano, T., Kosu, Y., and Kusumoto, M. Researches on ergot fungus. Part XIX. Correlation of sclerotinizability and alkaloid productivity of ergot fungus with its parasitic capacity. J. Agr. Chem. Soc. Japan, **24**, 471–476 (1951).
4. Abe, M., Yamano, T., Kozu, Y., and Kusumoto, M. Researches on ergot fungus. Part XX. Isolation of a mutant which produces good yields of agroclavine even in submerged culture. J. Agr. Chem. Soc. Japan, **27**, 18–23 (1953).
5. Abe, M., Yamano, T., and Kusumoto, M. Researches on ergot fungus. Part XXI. Production of ergot alkaloids in surface-replacement cultures (1). J. Agr. Chem. Soc. Japan, **27**, 613–617 (1953).
6. Abe, M., Yamano, T., and Kusumoto, M. Researches on ergot fungus. Part XXII. Production of ergot alkaloids in surface-replacement cultures (2). J. Agr. Chem. Soc. Japan, **27**, 617–620 (1953).
7. Abe, M., Yamano, T., Kozu, Y., and Kusumoto, M. Researches on ergot fungus. Part XXV. Production of alkaloids on *Elymus mollis* Trin. J. Agr. Chem. Soc. Japan, **29**, 364–369 (1955).
8. Abe, M., Yamano, T., Kozu, Y., and Kusumoto, M. Researches on ergot fungus. Part XXVI. Qualitative determinations of the ergot alkaloids present in the sclerotia and saprophytic cultures of ergot fungi. J. Agr. Chem. Soc. Japan, **29**, 697–703 (1955).
9. Baldacci, E. Production of alkaloids by ergot in agar culture. II. Pharmacological characteristics. Farmaco sci. e tec. **1**, 187–192 (1946); Chem. Abstr. **40**, 6668 (1946).
10. De Tempe, J. Alkaloidvorming door *Claviceps purpurea* Fries Tulasne in saprophytische cultur. Thesis, Univ. Amsterdam, 1945. (See Rev. Appl. Mycol. **25**, 210 (1946).)
11. Gjerstad, G. A note on the metabolism of lysergic acid by *Claviceps purpurea* (Fries) Tulasne. J. Am. Pharm. Assoc. Sci. Ed. **45**, 428–430 (1956).
12. Gjerstad, G. and Ramstad, E. Influence of certain chemicals on the growth and alkaloid formation of *Claviceps purpurea* (Fries) Tulasne in artificial culture. J. Am. Pharm. Assoc. Sci. Ed. **44**, 736–740 (1955).
13. Jaretzky, R. Alkaloidal content and activity of saprophytic ergot cultures. Arch. Pharm. **273**, 348–357 (1935); Chem. Abstr. **29**, 7013 (1935).
14. Kreitmair, H. and Küssner, W. The alkaloid content of *Claviceps purpurea* cultivated on an artificial medium. Biochem. Z. **239**, 189–192 (1931); Chem. Abstr. **25**, 5952 (1931).
15. McCrea, A. The reactions of *Claviceps purpurea* to variations of environment. Am. J. Botany, **18**, 50–78 (1931).
16. Macek, K., Cerny, A., and Semonsky, M. Mutterkornalkaloide. I. Die Bewertung von Ergotaminpräparaten mittels der Papierchromatographie. Pharmazie, **9**, 388–390 (1954).
17. Michener, H. D. and Snell, N. Studies on cultural requirements of *Claviceps purpurea* and inactivation of ergotamine. Am. J. Botany, **37**, 52–59 (1950).
18. Schweizer, C. Über die Kultur von *Claviceps purpurea* (Tul.) auf kaltsterilisierten Nährböden. Phytopathol. Z. **13**, 317–350 (1941).
19. Sim, S. K. and Youngken, H. W., Jr. The effects of certain organic and inorganic nitrogen feedings on growth and alkaloid biosynthesis in *Claviceps* (ergot) mycelium. J. Am. Pharm. Assoc. **40**, 434–439 (1951).
20. Sim, S. K. and Youngken, H. W., Jr. A study of growth effects induced in cultures of *Claviceps litoralis* Kawatani. J. Am. Pharm. Assoc. Sci. Ed. **43**, 429–432 (1954).
21. Stoll, A. von and Rüegger, A. Zur papierchromatographischen Trennung der Mutterkornalkaloide. Helv. Chim. Acta, **37**, 1725-1732 (1954).
22. Stoll, A. von, Brack, A., Kobel, H., Hofmann, A., and Brunner, R. Die Alkaloide eines Mutterkornpilzes von *Pennisetum typhoideum* Rich. und deren Bildung in saprophytischer Kultur. 36. Mitteilung über Mutterkornalkaloide. Helv. Chim. Acta, **37**, 1815–1825 (1954).
23. Taber, W. A. and Vining, L. C. A nutritional study of three strains of *Claviceps purpurea* (Fr.) Tul. Can. J. Microbiol. **3**, 1–12 (1957).
24. Tyler, V. E., Jr. and Schwarting, A. E. The culture of *Claviceps purpurea*. III. Tryptophan metabolism. J. Am. Pharm. Assoc. Sci. Ed. **43**, 207–211 (1954).